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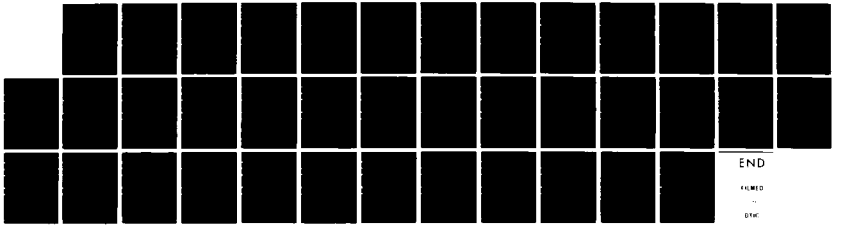

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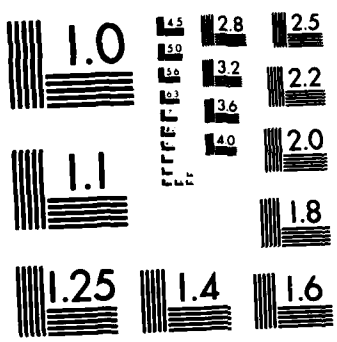

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Test of Suppressor Cell Activation in African Trypanosomes

Final Comprehensive Report

Arthur C. Zahalsky, Ph.D.

June, 1983

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Suppressor T-cells and macrophage activated in trypanosomiasis; Trypanosoma brucei S42; Trypanosoma Congolense; T-cell mitogen(s) released; trypanosomal phospholipases generate arachidonate; serum prostaglandins (PGE) elevated; indomethacin inactivates T-cell suppressors; PGE suppresses pfc response.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Suppressor cells from trypanosome infected mice are activated by exposure to trypanosome factor(s) in vitro. Primed, helper T-cells are not required for activation. The pfc response following removal of 'helpers' or 'suppressors' measures the activity of splenic suppressors from infected mice. Suppressor cell activation occurs in the absence of direct suppression of helper cells, or effector cell activation. Stimulation of prostaglandin (PGE) synthesis evokes suppressor cell activity, and is assayed by the effect of PGE_1, PGE_2, and PG inhibitors on the mixed spleen pfc response. (continued on reverse)

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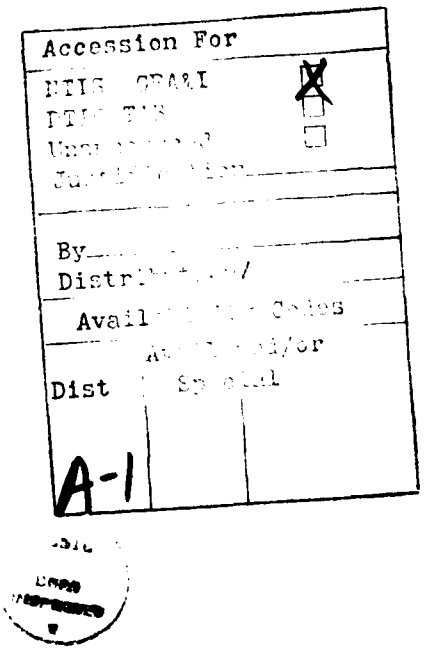
Block 20 (continued)

Glass adherent T-cells (GAT) added to non-adherent lymphocytes (NAL) at 24 or 48 hr. after initiation of culture suppressed DNA synthesis in NAL. The amount of PG (and metabolites) generated by NAL from initiation of culture to 48 hr. (by inhibition assay) appears to activate GAT in a manner analogous to that found when exogenous PGE_2 is added. Synthesis of PG in spleen cell cultures and in macrophage enriched populations suggests that the products of GAT and macrophage suppress DNA synthesis in NAL in vivo.



Summary:

Activation and expansion of T-suppressor cells (T_S), attributable to a T-cell mitogen released by pathogenic, bloodstream trypanosomes, was postulated to contribute in a major way to the development of immunosuppression in subacute murine trypanosomiasis. This hypothesis was tested using an in vitro system to determine whether product(s) of pathogenic trypanosomes exert a mitogenic effect on lymphocytes in culture. A postulated role for prostaglandins (PG) as mediators of immunosuppression, reducing lymphocyte responsiveness to activation by antigen, was also tested.

Initially, cell-free supernatant (TM_S) and pellet fractions (TM_p) of Trypanosoma brucei S42, and Trypanosoma congolense (strains obtained from WRAIR) were tested to determine whether such fractions could stimulate mitogenesis in cultured lymphocytes prepared from spleen cells of naive CE/J adult mice. The presence of T-cell mitogen in supernatant and pellet fractions was revealed by an increase in incorporation of tritiated thymidine (^{3}H-TdR) in glass-adherent lymphocytes (GAL), and most markedly in glass-adherent T-cells (GAT), which was found to exceed the increase in incorporation obtained with Conconavilin A (ConA), and phytohemagglutinin (PHA), the control mitogens. These results confirmed the earlier findings of Greenwood[1] that a T-cell mitogen is produced by pathogenic trypanosomes. Previously, only B-cell mitogenesis was known to occur in response to a trypanosome produced B-cell mitogen. The release of a T-cell mitogen by pathogenic, bloodstream trypanosomes briefly maintained in vitro under highly buffered conditions (usually, up to 18 hr), led to the use of TM_S and TM_p (hereafter referred to as 'Trypanosome Factor(s)') in assays of lymphocyte populations classified on the basis of differential expression of surface Ly-determinants (The conditions for use of the 'Trypanosome Factors' are identified in the body of the report as: 2 = Trypanosome Cell-free Soluble Factors, and 4 = TM_p). In these assays, conditions 2 and 4 were applied (introduced) to each of the five test conditions diagrammed in Figure 1. The five test conditions describe alternatives for assessing either a direct suppression by 'tryp. factor', or the direct activation of a suppressor cell population by 'tryp. factor'. The data obtained (for Fig. 1) support the latter interpretation, i.e. activation of a suppressor cell population.

The aforementioned findings led to the experiments described in Figure 2, which showed that the activated (expanding) suppressor cell population suppressed the plaque forming cell response (pfc) of normal spleen cells to Sheep Red Blood Cells (SRBC). Taken together, the findings from Fig's. 1 and 2 also indicated that a helper population (T_H) was not selectively activated by 'tryp. factor', nor was there an apparent requirement of primed (activated) helpers for activation of suppression.

[1]Greenwood, B.M. Possible role of a B-cell mitogen in hypergammaglobulinemia in malaria and trypanosomiasis. Lancet II. 435-436,(1974).

Next, spleen cells of infected animals (a counterpart of the assays shown in Fig. 2) were examined to determine whether activation of the suppressor population from the parasitized host occurs as a consequence of the presence of 'tryp. factors' (2 , 4) (the Control Design illustrated in Figure 3), or via a primed helper cell (the Experimental Design illustrated in Figure 4). The joint pfc results from Fig's. 3 and 4 indicated that suppressor cells from infected animals do not appear to require the help of primed helper T-cells to become activated, i.e. to suppress.

Do prostaglandins contribute as chemical mediators of suppression in trypanosomiasis? Would PGE added to spleen cells from normal, uninfected mice result in a mimicking of the reduced pfc response by spleen cells from infected animals? Would the pfc response of spleen cells from infected animals be increased following treatment with PG inhibitor? Do T-suppressor and T-helper cells, enriched by treatment with Anti-Ly antisera, produce PG?

The experiments outlined in Figure 5 address the first three questions. The findings support a role for PGE as a chemical mediator of suppression in trypanosomiasis. The data resulting from the addition of the cyclooxygenase inhibitor indomethacin lends additional support to this interpretation. A more difficult test is one which could ascribe a role to the T-suppressor cells as contributors to PG production (exclusive of any suppressor substance(s) released of protein or glycoprotein nature). The issue of PG production by suppressor cells was approached indirectly by the combined use of Anti-Ly antisera and indomethacin. The experiments outlined in Figure 6 were partially done (here the RO inhibitor compounds were not tested, hence condition d was omitted). The results obtained suggest that the Ly-2,3 subpopulation was a source of PG, and that helper cells from infected mice were neither a source of PG nor seemed to be involved in suppressing PG production.

Lastly, the source(s) of precursor for PG production by lymphocytes (and presumably macrophages) in infected animals was sought. We, and earlier others[2] found that autolyzing T. congolense (in vitro) release phospholipases A, and C. Phospholipase A_2 (PLA_2), assayed by the liposome method, was found to increase in amount as autolysis progressed. After 3 hr. no detectable arachidonic acid (A.A.) was found by high pressure liquid chromatography (HPLC). However, AA was found when trypanosome autolysate (no intact organisms) and rat red blood cells were incubated together overnight (14-18 hr). Elevation in the serum levels of PLA_2 and PLC in infected animals accompanied the increase in numbers of circulating trypanosomes, with marked increases in serum PGE_2 and PGF_2 following the rise in serum phospholipases. Serum PGE_2 and PGF_2 levels were determined by radioimmunoassay (R.I.A.). These levels increased to over 3500 pg/ml near the peak of parasitemia, in contrast to levels in non-infected mice of 600-780 pg/ml. Presumably, AA generated

[2]Roberts, C.J., Tizard, I.R., Mellors, A. and Clarkson, M.J. Lysophospholipases, lipid metabolism, and the pathogenesis of African trypanosomiasis. Lancet. 1187-1188, (1977).

by the action of trypanosome released PLA_2 and PLC deliver increased quantities of precursor for the synthesis of PG and other metabolites. By inference, PG production by expanded populations of T_S (and suppressor macrophages[3]) could act directly, through a second messenger or cAMP, to induce the release of a soluble suppressor substance.

The more critical test of the production of PG (and lipoxygenase pathway metabolites) by T_S from infected animals would be to supply radiolabelled AA to cultured cells, rapidly followed by HPLC analysis of the products (metabolites) in the culture supernates. We have not as yet done these experiments. The in vitro experiments detailed in Fig's. 5 and 6 do not distinguish between effects elicited by PGE and those attributable to other metabolites, possibly leukotrienes. The cell assay systems described in Fig's. 1-6 were not designed to identify or quantitate PG or LC metabolites generated by the cultured cells, not were the supernatants following the use of inhibitor(s) analyzed to determine whether such metabolites were altered, or removed. Exposure of Ly-defined T-suppressor cells to indomethacin does not rule out the possible synthesis and release of leukotriene metabolites whose effects may be anatagonistic to those commonly ascribed to PGE, or unknown. Merely noting that added PGE suppresses normal pfc response in the presence of inactivated (indomethacin exposed) suppressors does not exclude alternate pathway metabolites as possible direct or indirect suppressive molecules. These qualifications are noted here because the findings reported are to be taken cautiously as preliminary indications (though based on the accepted reliability of the pfc indicator system). The enormity of the tasks in the qualitative and quantitative analysis of PG and LC metabolites were then beyond our technical capacity and equipment facilities.

Additional findings, not within the scope of the work as originally proposed, and regretfully contributing to a lengthy delay in the preparation of this report, concern the presence and processing of mannose receptors of suppressor macrophage (Mϕ). Splenic suppressor Mϕ were operationally defined by their ability (and likewise, of T_S) to inhibit ConA mediated lymphocyte (T-cell) mitogenesis in culture. Splenic, alveolar, and peritoneal Mϕ from T. brucei S42 infected animals rapidly bind ^{125}I-beta glucuronidase. This binding is also present when the Mϕ are cultured overnight. Binding by freshly prepared and overnight cultured Mϕ is inhibited by mannan. Neither the number nor internal processing of mannose receptors in Mϕ from parasitized animals differed significantly from Controls (Mϕ from uninfected animals). Though Mϕ functionally capable of suppression did not appear to exhibit an increased binding capacity, these experiments should be repeated when other data are available comparing ^{3}H AA uptake and distribution of label in soluble and

[3]Morley, J., Bray, M.A., Jones, R.W., Nugteren, D.H., and van Dorp, D.A. Prostaglandin and thromboxane production by human and guinea-pig macrophages and leukocytes. Prostaglandins. 17. 730-736, (1979).

pellet fractions of Mϕ from control and infected animals.

Preliminary results on ^{14}C-TdR and ^{3}H-AA incorporation by peritoneal (P-Mϕ), splenic (S-Mϕ), and alveolar (A-Mϕ) may be summarized as follows:

(i) Normal PMϕ (uninfected, but zymosan elicited) showed a rapid uptake of AA into the pellet fraction, in contrast to the uptake noted for infected PMϕ. A steady increase (with time) in the appearance of label in the soluble fraction of normal PMϕ (and with a concommitant decrease of label in the particulate fraction) suggests some pooling of AA or metabolites. Label in the soluble fraction of infected PMϕ remained fairly constant, but there was a steady increase in the appearance of label in the particulate material from infected PMϕ, possibly due to the chasing of label from the pool, enhanced by the availability and saturation of unlabeled (endogenous) substrate in the infected animal. The sudden sharp increase in uptake of label after 24 hr in culture may be due to the depletion of endogenous reserves in culture.

Infected splenic Mϕ show a sudden, rapid uptake of AA into the pellet fraction, in contrast to the steady uptake noted for normal splenic Mϕ. A slight rise, followed by a shallow decline to previous levels, of label in the infected splenic Mϕ soluble fraction may indicate an increase in metabolite formation followed by a release of label (metabolites) into the culture medium (not determined). Normal SMϕ show a steady uptake of label into the particulate fraction, and a later increase in soluble label, presumably due to the synthesis and slight increase in metabolites (not determined).

Normal alveolar Mϕ show a rapid burst in the uptake of label, up to 5X that noted for PMϕ and SMϕ at 4 hr. This rapid uptake is followed by a steady decrease in the appearance of label in the particulate fraction. The soluble fraction of normal alveolar Mϕ shows a slight increase in label at 4 hr and a return to the previous (baseline) level by 16 hr. However, the amount of label is 3-4X that noted for normal PMϕ and normal SMϕ at 4 hr, but with depletion by 16 hr. to comparable levels. Infected AMϕ showed a moderate increase in the appearance of label in both the particulate and soluble fractions at 4 hr. By 16 hr. there was significant accumulation of label in the particulate fraction accompanied by a return of label in the soluble fraction to the level noted at 1 hr. These results would seem to be consistent with a proposed release of label (metabolites?) from these cells to the medium (not determined).

Where precipitous declines in both particulate fraction incorporated label and soluble label occurred such declines were attributed to cell destruction, as noted by a correlate decline in ^{14}C-TdR incorporation.

Contaminating cells in the SMϕ and PMϕ preparations provide a source of debris for proteolytic enzymes under culture conditions where serum is omitted from the medium. The higher degree of purity of the AMϕ could account for thr reduced viability of these cells in culture, apparently due to the release of proteases by autolysed Mϕ.

There are several criticisms of these preliminary findings, notably: we have not determined that AA metabolites were generated and released from the cytosol to the medium. This would require that the media be extracted, and analyzed by HPLC. Also, label incorporated into the particulate fraction was not identified by its presence in isolated phospholipids. Finally, since PG and LC metabolites are generally extremely short-lived, it would be necessary to extract the culture media most rapidly (under N_2) to preserve the metabolites. The aforementioned are requirements to be met for proper interpretation of the data.

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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COMMENT

The conclusions enumerated below are supported by the data that follows. Please refer to Figures 1-6 (each figure corresponds to the same numbered text section, e.g. Section 1 refers to Fig. 1 protocols) for findings. Collectively, these data indicate that:

(i) trypanosome cell-free soluble factor(s) (condition [2], Fig. 1) and a sonicated pellet fraction from trypanosomes (condition 4, Fig. 1) induce suppressive activity.

(ii) as a consequence of treatment with [2] or [4], T-helper cells are activated (mitogenesis occurs),

(iii) T-effector cells (cytotoxic, T_c) appear to be suppressed in response to [2] or [4], and

(*iv) Activation of T-suppressor cells (T_s) occurs in response to [2] or [4].

*T_s activation data are consistent with expectations for cell phenomena occurring during immunosuppression in trypanosomiasis. These data also underscore the need to investigate the metabolism and products of suppressor cells in parasitemia's where immunosuppression occurs.

Section 1

1. Treatment with anti-θ+ C'

Tubes 6,7, and 8 showed an absence of lymphocyte activation (no significant incorporation of label). These control tubes indicated that the responsive cells in tubes 2-4, 10-12, 14-16, and 18-20, were T-cells.

2. Evidence of direct suppression by treatment with [2] and [4].

In response to treatments [2] and [4] suppression of spleen cell responsiveness to ConA stimulation occurred. Given these data, the following results revealed whether the subpopulations of T-cells were suppressed.

A. Suppression of T-helper cells (T_H).

Direct suppression of helper cell activation was <u>not</u> indicated by the data in tubes 14-16 (which contained an enriched population of T_H). As noted, the population of T_H was responsive to ConA activation in tube 15.

B. Suppression of T-effector (cytotoxic, T_c) cells.

(i) Suppression of effector cell activation was suggested by the data in tubes 18-20, containing an effector population.

The results in tube 19 indicated that a trypanosome derived factor suppressed effector cell activation by ConA.

(ii) Selective suppression of T-effectors was not indicated since tubes 10, 11, 12 (containing effectors & suppressors) and 14, 15, 16 (T_H) did not show the data anticipated. However, the results in tube 11 indicated that in the population of suppressors plus effectors, one or both subpopulations could be activated by ConA in the presence of trypanosome factor. The activated subpopulation was substantiated as suppressors by the data for tubes 18-20. The data for tube 15 indicates that T_H were not suppressed in their response to ConA activation.

3. Evidence for T-suppressor cell activation by treatment with [2] and [4].

Suppression did not occur directly, i.e. by a trypanosome derived factor, but seemed to be due to suppressor cell activation. This latter interpretation was suggested by the data noted in tubes 2, 3, and 4. The elevated response to trypanosome factors in tube 1 indicated that [2] and [4] activated some T-cell subpopulations (not shown is a control with no treatment, which represents the base line activity of the mixed spleen cell population). This control tube #1 is illustrated along with identical tubes for each experimental condition (1,5,9,13,17). For convenience, these tubes are not recorded since they all contained few counts above background.

A. Activation of T-helper cells

(i) Activation of T_H was indicated by the data in tubes 14-16. These results show (since the population in tube 14 was activated) that the trypanosome factor(s) tested were mitogenic for T_H. Thus, tube 14 mimics the results of its positive control, tube 15, which was treated with ConA.

(ii) Selective activation of T_H was not indicated. The mixed populations of T_s and T_c (tube 10) were activated and the population of effectors (tube 18) was activated in response to trypanosome derived factor(s). Thus, selective activation of T_H is not mediated by a failure to activate T_s in the absence of primed T_H.

With these data at hand, the experiments next described tested whether activation of suppressor cells occurred by primed T_H, unprimed T_H, or neither.

B. Activation of T-effector cells (T_c)

(i) Direct activation of T_c was not found, i.e. the data from tube 18 showed reduced cpm, whereas high incorporation would have been anticipated were the T_c to have been directly activated. Activation of T_c, especially selectively, was not anticipated since suppression would not result. However, since

a cell-mediated immunity has been reported in trypanosomiasis, activation of T-effectors by some means is to be reconciled with the data presented here.

C. Activation of T-suppressor cells (T_s).

(i) Activation of T_s was inferred from the data comparing activation among tubes 10, 11, 12 with those among 18, 19, and 20. Tubes 10-12 contained T_s and T_c, and 18-20 contained only an effector population. Direct activation of suppressor cells by trypanosomal factors [2] and [4] was suggested by the fact that the population of suppressors plus effectors were activated, but this activation did not occur in the effector population alone.

(ii) Selective activation of T_s was inferred from the data of tubes 10-12, with apparent activation of T_H (noted by the high levels of incorporation in tubes 14-16).

Since activation of a T_s population was noted, the following experiments were done to examine the ability of the suppressor population to suppress the PFC (plaque forming cell) response of normal spleen cells.

Section 2

1. Activation of Suppressor Population(s) by Trypanosome Derived Factor(s)

The data from the protocols shown in Fig. 1 revealed that trypanosome derived factor(s) activate a suppressor cell population. The experiments detailed in Fig. 2 were done to determine whether these activated cells suppressed the pfc response of normal spleen cells to sheep red blood cells (SRBC).

Control plates A_1 and A_3 both showed a reduced pfc response in comparison to the normal control, plate A_2. On plate A_1 the suppressor was activated by ConA nonspecifically, and plate A_3 contained trypanosome activated suppressors (these findings do not permit the inference, however, that <u>direct</u> activation of suppressor cells occurred). Activation of a suppressor population is supported by plate G which showed a reduced pfc with respect to plate E (the latter contained the normal response of untreated suppressor cells). Plate G data indicated that the response of suppressor cells to trypanosome derived factor(s) under conditions [2] and [4] mimicked the reduced pfc response of ConA activated suppressors (plate F).

2. Requirement of Primed Helpers for Suppression of Normal PFC Response.

Since the data from Section 1 indicated that a helper population was activated by treatment with trypanosome factor(s),

the experiments detailed in Fig. 2 were done to test the requirement of primed (activated) helper's for activation of suppression. As in '1' above, suppression was tested as a reduced pfc response of normal spleen cells to SRBC.

Plates A_1, A_2, and A_3 (activation of suppressor cells) as control plates yielded similar results. Nonspecific ConA activation of a suppressor population yielded a reduced pfc to SRBC (plate A_1), in comparison to untreated spleen cells (A_2). Treated spleen cells (factors [2] and [4]) yielded a reduced pfc and appeared to mimic ConA treated spleen cells in this respect. However, whether ConA treatment or treatment with trypanosome factor(s) activated a suppressor population via the helper cell was not discernible from the results obtained in the control plates.

The requirement of a primed helper cell for suppressor activation was tested by mixing ConA and trypanosome factor activated (primed) helper cells with normal, untreated suppressor cells. A comparison was made of the resultant pfc response to that generated by unprimed helpers plus normal, untreated suppressor cells.

Since plate D showed a significantly reduced pfc response similar to plates A_1, A_2 and F, a primed helper appeared not to be required for activation of suppression (Plate D contained the pfc response of unprimed helpers plus normal, untreated suppressors). Plate C served as a positive control for plate B, since the helper cells here were activated by ConA; thus, B mimicked the pfc response of C. The involvement of primed cells in the activation of suppression was not found.

Since activation of the suppressor population as a consequence of interaction with trypanosome derived factor(s)[2]and [4]seemed to occur, it was necessary to examine the spleen cells of infected animals. These experiments are outlined in Fig. 3.

Section 3

1. Suppressor Cell Activity in Trypanosome Infections

Figures 2 and 4 illustrate the protocols used to test the 'nature' of the suppressor cell in trypanosome infected mice. Figure 3 shows the necessary controls for the 'variable testing tubes' noted in Fig. 4.

A. Control Design (Fig. 3)

The normal pfc response of spleen cells from uninfected animals was tested on Plate A. To demonstrate that this mixed spleen population contained no helper or suppressor populations which were suppressive by themselves, plate B containing suppressors, and plate C containing a helper population were tested. These plates showed a normal anti-SRBC pfc response.

Plate F was included to show a pfc response of mixed spleen cells from infected animals. This response was reduced compared to normal (Plate A). Plates D, and E tested the pfc response of the suppressor and helper populations respectively, from infected animals. Plate D, containing activated suppressors, showed a reduced pfc response, mimicking that seen for the mixed spleen population plate from infected animals (Plate F). Plate E, containing a helper population from infected animals (suppressors removed) generated a near normal anti-SRBC compared to plates A and C.

B. Experimental Protocols (Fig. 4).

Figure 4 protocols tested whether the suppressor cell population required the help of primed T_H cells to become functionally activated.

Plates G and J were controls for normal, uninfected spleen cells, and normal, infected spleen cells, respectively. Plate G was actually identical to A in Fig. 3, and Plate J was the same as Plate F in Fig. 3. It was not necessary to repeat plates G and J for the given spleen preparation, eventhough these were noted in Fig. 4.

Plates H and I tested whether the suppressor population in infected mice required a primed helper for activation. Plate H pfc response was not reduced with respect to plate G, i.e. unactivated suppressors from uninfected animals, and primed helpers from infected animals did not suppress an anti-SRBC pfc response.

Plate I denoted the ability of suppressors from infected animals and helpers from uninfected animals to suppress the pfc response of normal spleen cells. Since this mixed population of spleen cells contained activated suppressors from infected animals, the pfc response mimicked plates J and D (activated suppressors only) and were reduced with respect to plate G. Plate I tested whether unprimed helpers had any effect on a previously activated suppressor population. Such did not appear to be the case.

Having tested the requirements of primed helper cells in this activation process, we next directed our attention to a study of the suppressor substance(s) in trypanosomiasis.

Sections 4,5,6.

1. Effects of Prostaglandins E_1 and E_2 and Prostaglandin Inhibitors on Mixed Spleen Cell pfc Response.

To test the possibility that prostaglandins were the chemical mediators of suppression in trypanosomiasis, spleen cells from the uninfected and infected animals were treated with PGE_1 and

PGE_2 and PG inhibitors, and the resultant pfc responses were measured.

A. Figure 5 describes the first of these experiments. Here, pfc response A is the normal response of spleen cells. Plate A_2 was repeated 1x, each treated with a) PGE_1, b) PGE_2, c) Indomethacin, and d) R compounds, respectively. The response of plate A_2 to treatment (a) or (b) mimicked the reduced pfc response of plate J_1 (spleen cells from infected animals). The pfc response of plate A_2 treated with PG inhibitors was not significantly altered.

However, the pfc response of plate J_2, (spleen cells from infected animals) was not significantly increased upon treatment with PG inhibitors, (c) and (d). Plate J_2 in this case did not show significant further reduction in pfc response upon treatment with exogenous PG's, (a) and (b).

In summary, the data in Section 4,A supports the hypothesis that the chemical suppressing agent(s) in trypanosomiasis may be PGE_1 and/or PGE_2, as evidenced by data showing that:

(i) treatment of spleen cells from uninfected animals mimicked the pfc response of spleen cells from infected animals (Plate A_2, a or b treatment mimicked plate J_1).

and (ii) treatment of spleen cells from infected animals restored the pfc response to near normal levels (Plate J_2 c or d treatment mimicked plate A_1).

B. To test whether a T suppressor population produced prostaglandin(s), the experiments outlined in Fig. 6 were done. Here, as in Figs. 3 and 4, spleen subpopulations were enriched by treatment with Anti-Ly-antisera, and these cells were then treated as described above with PGE_1, PGE_2, and PG inhibitors.

As noted under 'A' above, plates A_1 and J_1 were untreated controls for uninfected and infected animals respectively.

Plates B_1 and B_2 tested whether exogenously supplied prostaglandins suppressed normal pfc responses in the presence of inactivated suppressors. Plate B_1, treated with PG (a), and (b) showed a reduced pfc with respect to plate B_2, untreated control, and Plate A_1. Plates B_1 and B_2, as expected, did not show an altered pfc in response to treatments (c) and (d), but plate D_1 showed a significantly increased pfc with respect to plate D_2. Plate D_2 contained the pfc response to suppressors from infected animals, and PG inhibitors, as expected, restored the pfc response of these cells on plate D_1. These results are supported by the fact that PG inhibitors did not significantly alter the pfc response of plate B_1 with respect to B_2, and addition of PG's only

slightly accentuated the reduced pfc seen on plate D_1 with respect to D_2.

To test that the subpopulation of T cells, Ly 2,3, were the sole source of suppressive PG's, plates C_1, C_2, E_1 and E_2 were done to test the pfc response of the helper population. Plate C_1 was unaffected by PG inhibitors (c) and (d), and showed a marked reduction in pfc response with respect to C_2 when treated with added PGE_1 and PGE_2. These results were anticipated because these plates tested helper cells from uninfected animals.

Plate E_1 mimicked the response of plate C_1, indicating that helper cells were not involved in suppressing prostaglandin production, since these helper cells were taken from infected animals.

SIGNIFICANCE - SECTIONS 1-6

Some objectives of this project are addressed by the results noted for Figures 1-6. These results indicate that:

1. a suppressor cell line appears to be activated after exposure of splenic cells to trypanosome factor(s) in vitro (Figure 1). The incorporation of ^{3}H-TdR supports this conclusion.

2. a requirement of primed helpers for suppressor activation is not seen (Figure 2). The pfc response to SRBC supports this conclusion.

3. splenic suppressor cells are detectable and their activity measureable in trypanosome infections (Figures 3 and 4). The pfc response following removal of helpers or suppressors from uninfected or infected mice supports this conclusion.

4. The effect of PGE_1, PGE_2 and PG inhibitors on the mixed spleen cell pfc response, and their effects on enriched helper and suppressor subpopulations from uninfected mice (Figure 6) may be compared and measured.

The work described here has distinguished between the assertions that pathogenic trypanosomes evoke suppressor cell functions (by stimulating PG synthesis in suppressor cells), and suppression attributable directly to trypanosome products. The former activity is indicated by experiments which showed suppressor cell activation in the absence of direct suppression of helper cells, or effector cell activation.

SECTION 7

Comment

The experiments described in Section 7 extend those reported in Sections 1 through 6, and are similar in design to those described in Figures 1 and 5. The data in Sections 4-6 support a role for prostaglandins in the regulation of lymphocyte activation in the parasitized host. The data in Section 7 indicate that prostaglandins evoke suppressor populations in trypanosomiasis.

Materials & Methods

The separation of spleen cells into adherent (GAL) or non-adherent (NAL) lymphocyte fractions on glass wood columns was accomplished as described by Webb and Jamieson[1]. These populations were analyzed by cytotoxic antisera, i.e. the NAL were composed of 80-85% T cells, 15-20% B cells, with <5% macrophage; GAL were 60-70% B cells, 30-40% T cells, with <5% macrophage. GAT were prepared by incubating GAL with an appropriate dilution of rabbit anti-mouse μ chain specific antiserum in the presence of guinea pig complement for 1 hr @ 37°C (with gentle stirring). The cells remaining were washed extensively in HBSS and counted using the trypan blue exclusion method (to assess viability). This cell population was composed of $\sim$95% T cells (based on treatment with specific B cell mitogen and antithymocytic antiserum.

Splenic macrophages were prepared by incubating whole spleen cell suspensions in MEM + 10% heat inactivated FCS for 30 min. on plastic petri's at 37°C. The plates were rinsed 3x with warm HBSS and the adherent cell (Mϕ) removed by gentle scraping. The cells were counted by the hemocytometer method and stained with trypan blue.

Subfractionation of NAL on Sephadex G-10 was carried out using the method Ly and Mishell[2]. The presence of macrophages was determined on the basis of adherence to glass.

*Abbreviations used: GAL(Glass Adherent Lymphocytes): GAT(Glass Adherent T lymphocytes); NAL (Non-adherent Lymphocytes): MAC (Macrophages - splenic); PG (Prostaglandin); PGSI (Prostaglandin Synthetase Inhibitor); TM-I (mitogen from derived cell-free fractions).

[1]Webb, D.R. and Jamieson, A.F., Cell Immunol. 24, 45 (1976)
[2]Ly, L. and Mishell, R.L. J. Immunol. Methods 5, 239 (1974).

Splenic lymphocytes were cultured in COSTAR microtiter trays in RPMI 1640 + antibiotics + glutamine. The concentration of lymphocytes/well was adjusted to 5×10^5 or 1×10^6. Sterile 0.1M Hepes was added to each well. The dose range of mitogen (ConA) was 0.5 - 2.0 µg/ml, corresponding to the dose range for optimal stimulation of DNA synthesis when PHA was used as mitogen. Radiolabel (1µCi - ^{3}HTdR) was added at 60 hr. Cells were harvested at 72 hr. by trapping on 0.45µ Millipore filters. The filters were washed with 5% TCA, dried, placed in scintillation vials containing 10 ml scintillation cocktail, and the vials counted.

Findings

PGE_2 inhibited DNA synthesis in NAL (measured after 72 hr) when added up to 48 hr after culture initiation. TM-I stimulated DNA synthesis in NAL from uninfected animals, but markedly less so in NAL from infected mice. PGE_2 also inhibited DNA synthesis when the PG was added after 24 hr. Similar results were obtained when TM-I was added after 24 hr under the same conditions (see Table 1).

To contrast the effect of delayed vs immediate addition of PGSI on ConA or trypanosome-mitogen stimulated cultures of NAL/GAL, indomethacin was added to cultures of NAL/GAT (both infected and uninfected) in which the relative concentration of GAT to NAL was varied. Indomethacin blocked GAT suppression, relative to the concentration of GAT cells present. No reversal of GAL suppression was obtained at a high ratio of GAL/NAL when RO-20-5720 or indomethacin was added at 24 or 48 hr. after initiation of culture (with lymphocytes from uninfected animals). In contrast, some reversal of suppression occurred when these inhibitors were added at the time of culture with NAL from infected animals but GAL from uninfected mice (Table 2).

The effect of NAL on GAT suppressor cell activation was determined. PG, if produced by both NAL and GAT, might regulate suppressor cell function. Populations of T suppressors (GAT) were exposed to ConA, TM-I, PGE_2, or ConA + PGE_2 for 48 hr in the presence or absence of PGSI; or TM-I + PGE_2 for 48 hr in the presence or absence of PGSI. After 48 hr. the supernates and pelleted organisms were tested for their ability to suppress DNA synthesis in NAL. GAT were exposed to ConA, PGE_2 (10^{-6}M) or both, and TM-I, PGE_2 (10^{-6}M) or both for 48 hr (cells were centrifuged, resuspended in fresh medium, counted, viability determined, mixed in varying ratios with fresh NAL and exposed to mitogen for 72 hr, the assayed for ^{3}H-TdR incorporation). The GAT showed enhanced suppressor ability (Table 3).

Supernates were collected from GAT exposed to ConA, PGE_2, or both, and TM-I, PGE_2 or both in the presence or absence of PGSI,

after 48 hr. in culture. These supernates were mixed with NAL stimulated with either ConA or TM-I. The supernates obtained from GAT exposed to PGE_2, ConA + PGE_2, or TM-I + PGE_2, showed the most suppressor activity.

To determine the effect of macrophages and PGSI on DNA synthesis in NAL, the NAL were cultured with varying concentrations of splenic macrophages (from infected and uninfected animals) in the presence or absence of RO-20-5720. In these experiments the ratio of NAL/macrophage was adjusted so as to be comparable to the ratio of NAL/GAT. The data support the interpretation that 'suppressor' macrophages are present in the spleen of the parasitzed mice (Table 4).

Significance

The ability by trypanosome mitogen to stimulate synthesis of PG in spleen cell cultures and in macrophage enriched populations suggests that GAT and macrophage products suppress DNA synthesis of NAL in vivo. Endogenous synthesis of PG in GAT is thought to 'trigger' events which result in the profound immunosuppression seen during trypanosome parsitemia. At an appropriate level of serum PG (from GAT suppressors) suppression occurs. This view is supported by findings showing that suppressor cells (GAT) added at 24 or 48 hr. after culture initiation still suppressed DNA synthesis in NAL, suggesting that at 24 or 48 hr. PG was at a level sufficient to evoke suppressor cells, i.e. the amount of PG (and metabolites) generated by NAL from initiation of culture up to 48 hr. (by inhibition assay) activated GAT in a manner analagous to that found when exogenous PGE_2 was added. These results do not exclude other products (metabolites) of the PG synthetase pathway as possible mediators, especially since PG metabolites[3] have been found to be highly effective at stimulating cAMP levels[3]. These findings also indicate that mitogenically activated, non-specific suppressor cells, elicited in response to trypanosome mitogen, are present in both lymphocyte and macrophage enriched cell populations.

[3]Gorman, R.R., Bunting, S. and Miller, O.J., Prostaglandins 13, 377(1977).

Table 1.

Purpose: Effects of PG on ConA and Trypanosome Mitogen Stimulated DNA Synthesis in NAL.

Description: Others have shown that PGE_1 or PGE_2 additions at the time of initiation of culture inhibited transformation by mitogen[4,5]. PGE_2 was added at the time of culture initiation and at various times thereafter. In some cases a prostaglandin synthetase inhibitor (PGSI) was added to the cultures at initiation to prevent PG biosynthesis.

	Condition in Culture	Time at Addition	CPM±SE[1] (Con-A)	CPM±SE[1] (TM-I)
(i)	ConA@1µg/ml or TM-I@ 50λ	--	34,387 ±2724	23,481 ±1792
(ii)	ibid +$PGE_2 10^{-6}$M	0	5,215 ±886	4,272 ±703
(iii)	ibid +$PGE_2 10^{-7}$M	0	**7,404 ±1019	6,316 ±887
(iv)	ibid +$PGE_2 10^{-6}$M	24	4,105 ±619	3,872 ±563
(v)	ibid +$PGE_2 10^{-6}$M	24	**8,664 ±1423	7,911 ±1202
(vi)	ibid +$PGE_2 10^{-6}$M	48	14,824 ±2887	14,212 ±2677
(vii)	ibid +$PGE_2 10^{-7}$M	48	24,489 ±1623	23,925 ±1604
(viii)	ibid +$PGE_2 10^{-6}$M	70	**31,119 ±1017	30,286 ±973
(ix)	ibid +$PGE_2 10^{-7}$M	70	32,775 ±2626	31,427 ±1972
(x)	ConA@ 0.5µg/ml or TM-I@ 50λ +RO-20-5720 10^{-6}M		144,691 ±5218	137,385 ±4861
(xi)	ConA@ 0.5 µg/ml or TM-I@ 50λ +$PGE_2 10^{-6}$M	0	92,406 ±3872	91,211 ±3717
(xii)	ibid +$PGE_2 10^{-6}$M	24	95,515 ±4102	92,388 ±3701
(xiii)	ibid +$PGE_2 10^{-6}$M	48	123,573 ±4108	125,347 ±5114

[1]Values represent the mean CPM of either triplicate or quadruplicate cultures exposed to tritiated thymidine (^{3}H-TdR) for 4 hr (i-ix) or 21-22 hr (x-xiii).

**Results obtained with PHA parallel those for ConA.

[4]Peulus, L.M. and Strausser, H.R., Life Sciences 20, 903(1977).
[5]Parker, C.W., Bauman, M.L. and Huber, M.G., J. Clin. Invest. 52, 1336(1973).

Table 2.

Purpose: Effect of Delayed vs Immediate Addition of PGSI on ConA or Trypanosome Mitogen stimulated Cultures of NAL/GAT.

Description: The competitive inhibitor, indomethacin was added to cultures of NAL/GAT (from both infected and uninfected animals) in which the relative concentration of GAT to NAL was varied. The ability by indomethacin to block GAT suppression, relative to the concentration of GAT cells present, was noted.

	Cells in Culture		Source+	^{3}H-TdR Incorporated c̄ Indomethacin++	c̄/o Indomethacin
(i)	NAL		UA	49,586 ±4973	42,337 ±4012
(ii)	NAL		IA		
(iii)	NAL/GAT***	4:1	UA	9,223 ±1518	5,779 ±346
		8:1	UA	19,178 ±2170	10,183 ±1476
		16:1	UA	31,256 ±3208	18,275 ±2081
		32:1	UA	34,109 ±1956	23,871 ±2423
		64:1	UA	36,883 ±2714	24,889 ±2855
(iv)	NAL/GAT	4:1	UA/IA	6,103 ±1127	2,411 ±288
	NAL/GAT	16:1	UA/IA	11,447 ±1751	6,012 ±1226
	NAL/GAT	64:1	UA/IA	20,812 ±3189	14,223 ±1819

+UA (uninfected animals); IA (infected animals)
*ConA @ 0.5 µg/ml; ++indomethacin, 10^{-7}M, at start of incubation period
***GAT added at various dilutions c̄ $1x10^6$ NAL/well

Table 3.

Purpose: Effect of NAL on GAT Suppressor Cell Activation.

Description: PG, if produced in both NAL and GAT cells could be regulating the functioning of the suppressor cell. To test this presumption populations of T-suppressor's (GAT) were exposed to ConA, TM-I, PGE_2, or ConA + PGE_2 for 48 hr in the presence or absence of PGSI, or TM-I + PGE_2 for 48 hr in the presence or absence of PGSI. After 48 hr. the supernatants and pelleted organisms were tested for their ability to suppress DNA synthesis in NAL. These data contrast the effects of exogenously added PG on the function of suppressor cells from parasitized and uninfected animals.

	Ratio of NAL:GAL	GAT EXPOSED TO	^{3}H-TdR Incorp.
(i)	4:1	NAL+ConA or NAL+TM-I	22,516 ±2218
(ii)	8:1	ibid	47,318 ±4661
(iii)	16:1	ibid	65,119 ±5172
(iv)	32:1	ibid	73,634 ±6109
(v)	64:1	ibid	89,826 ±6833
(vi)	4:1	NAL+ConA+RO-20-5720*	24,420 ±1917
(vii)	4:1	NAL+TM-I+RO-20-5720	20,008 ±1482
(viii)	8:1	ibid	54,208 ±4943
(ix)	16:1	ibid	72,027 ±5337
(x)	32:1	ibid	86,503 ±6114
(xi)	64:1	ibid	119,221 ±7652

*RO-20-5720 @ 10^{-6}M

Table 4.

Purpose: Effect of Macrophages and PGSI on DNA Synthesis in NAL.

Description: Macrophages produce prostaglandins and are known to contribute to the regulation of lymphocyte function[6,7]. It is possible that even low levels of macrophage in the experimental data in Tables 1-3 influenced the results obtained. Therefore, NAL were prepared and cultured with varying concentrations of splenic or peritoneal macrophages obtained from infected and uninfected animals, in the presence or absence of RO-20-5720.

Conditions in Culture+	RO 20-5720*	^{3}H-TdR Incorp.**
(i) NAL	+	53,668 ±4716
	-	39,423 ±3448
(ii) NAL + 1% Mϕ (UA)	+	59,275 ±4825
	-	37,713 ±3664
(iii) NAL + 1% Mϕ (IA)	+	66,163 ±5088
	-	41,166 ±3712
(iv) NAL + 10% Mϕ (UA)	+	66,085 ±6014
	-	37,122 ±3306
(v) NAL + 10% Mϕ (IA)	+	82,707 ±5941
	-	40,236 ±3817
(vi) NAL + 20% Mϕ (UA)	+	60,004 ±5119
	-	38,553 ±3549
(vii) NAL + 20% Mϕ (IA)	+	89,811 ±6635
	-	41,473 ±4008

*RO 20-5720 @ 10^{-6}M
**Incorp. after 21-22 hr.
+Mϕ @ 5 x 10^4 - 2 x 10^5/culture

[6]Waksman, B.H. and Namba, T., Cell Immunol. 21, 161(1976).
[7]Zimecki, M., and Webb, D.R., J. Immunol. 117, 2158(1976).

Production of Ly-antisera

The immunoregimen noted in chart form below was used. Fourteen inoculations spaced over a 7-month period yielded useful titres of Ly-antisera.

Specificity*	Recipient	T-Cell Antigen Phenotype of the Recipient	Thymocyte Donor	T-Cell Antigen Phenotype of the Donor
anti-Ly 1.2	C3H/He	1.1	CE/J	1.2
anti-Ly 2.1	B6/H2^{R}**	2.2	CE/J	2.1
anti-Ly 3.2	C58	3.1	CE/J	3.2

* Phenotypically matched at other loci

** C57Bl/6 x AKR/J F_1 substitutes for B6/H2^{K}

DATA: Figure 1: Direct Suppression or Direct Activation of a Suppressor Cell Line. (Incorp. of ^{3}H-Thymidine (1μCi) measures mixed lymphocyte response ć either 2.5 x 10^5 or 5 x 10^5 cells).

Tube #	CPM (^{3}H-TdR)*±SE
1	NR
2	34,123±1214
3	49,284±2123
4	21,579±1017
5	NR
6	1104±93
7	537±61
8	728±74
9	NR
10	16,836±1138
11	23,714±1274
12	14,106±966
13	NR
14	67,135±3478
15	88,810±4059
16	43,117±2821
17	NR
18	4,921±432
19	4,345±578
20	3,829±513

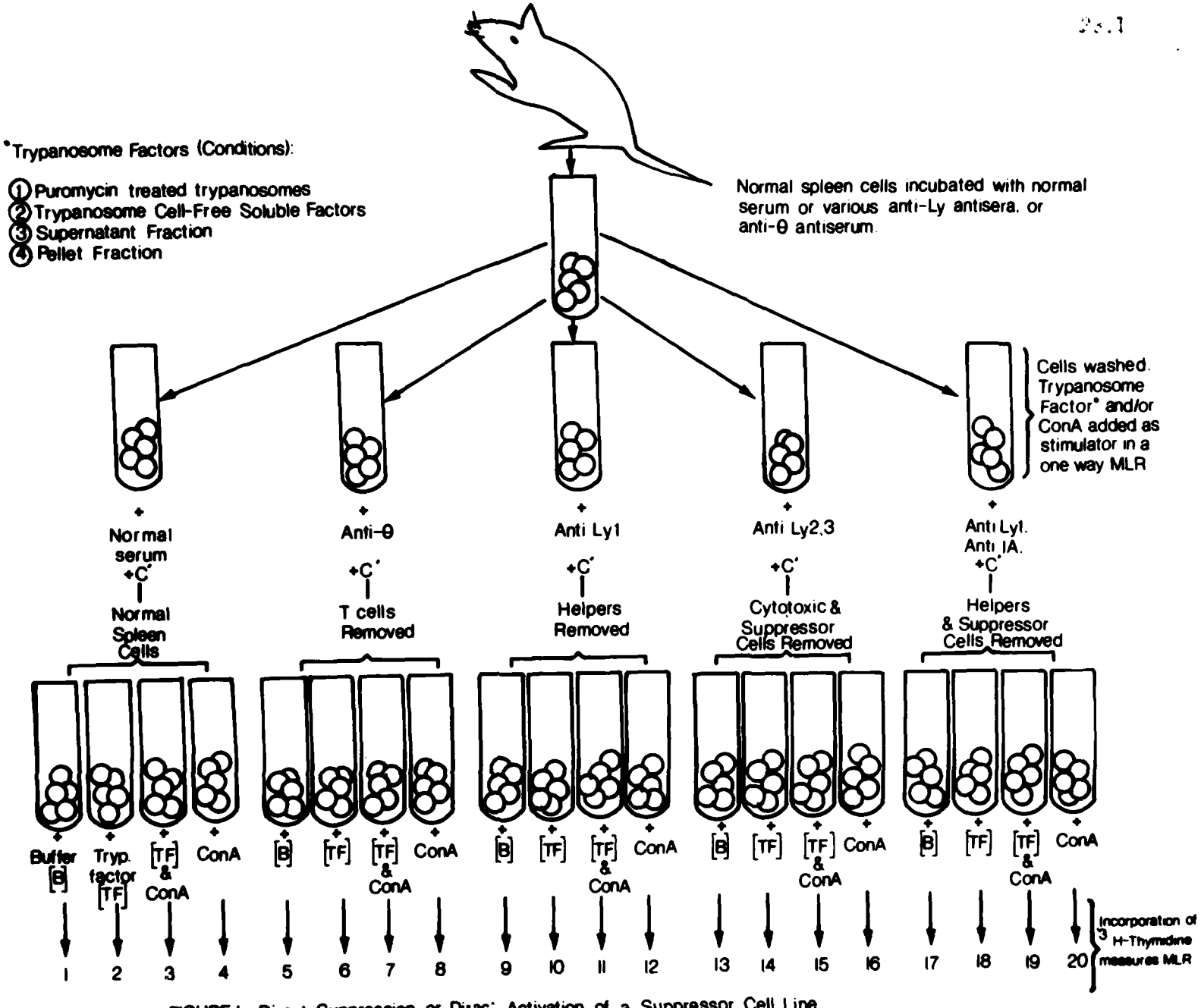


FIGURE I. Direct Suppression or Direct Activation of a Suppressor Cell Line

DATA: Figure 2: Requirement of Primed Helpers for Suppressor Activation.

Plate Designation	PFC
A_1	513
A_2	842
A_3	487
B	679
C	713
D	461
E	772
F	505
G	614

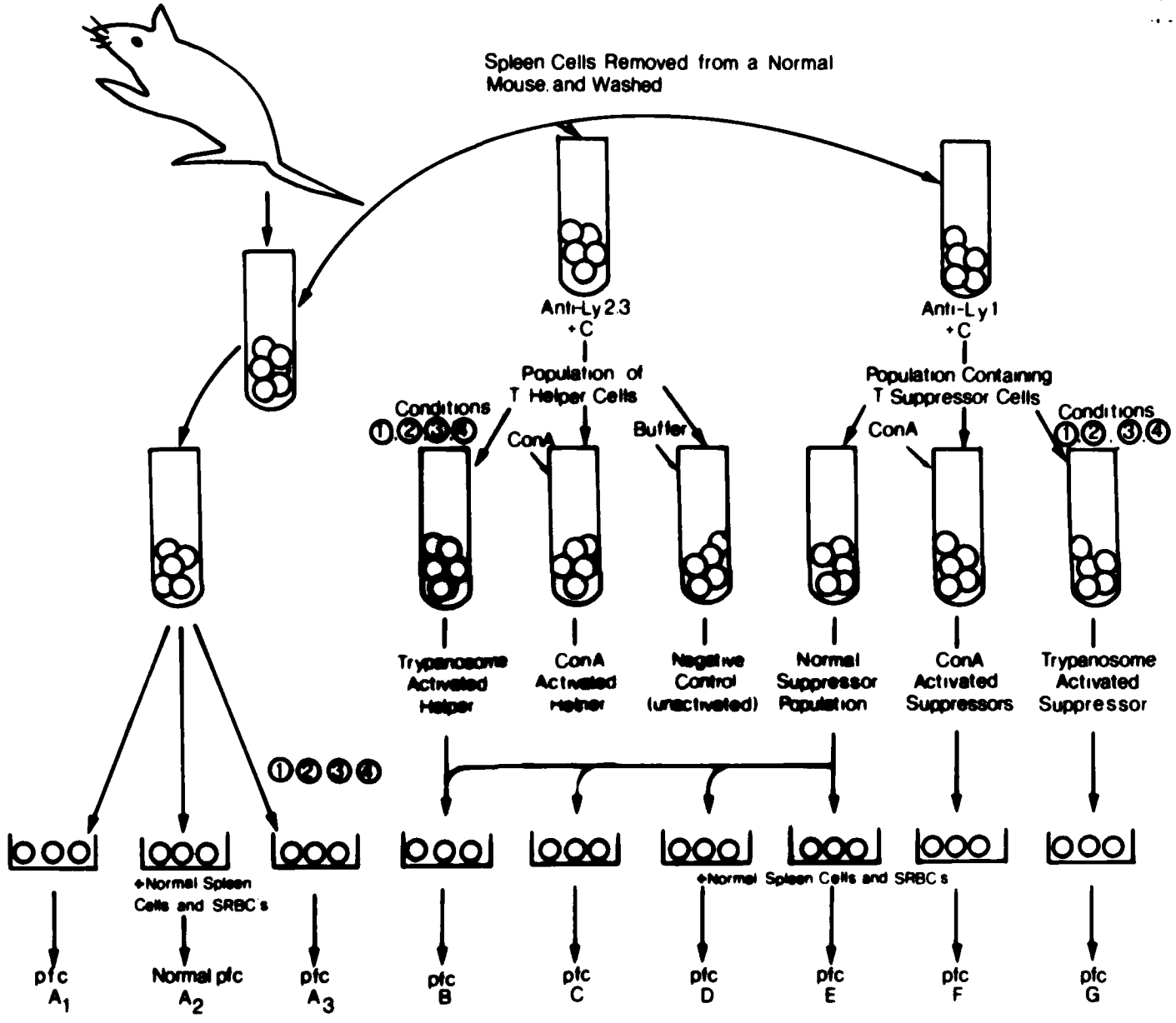


FIGURE 2 Requirement of Primed Helpers for Suppressor Activation

DATA: Figure 3: Suppressor Cells in Trypanosome Infections

Plate Designation	PFC
A	823
B	795
C	841
D	466
E	714
F	439

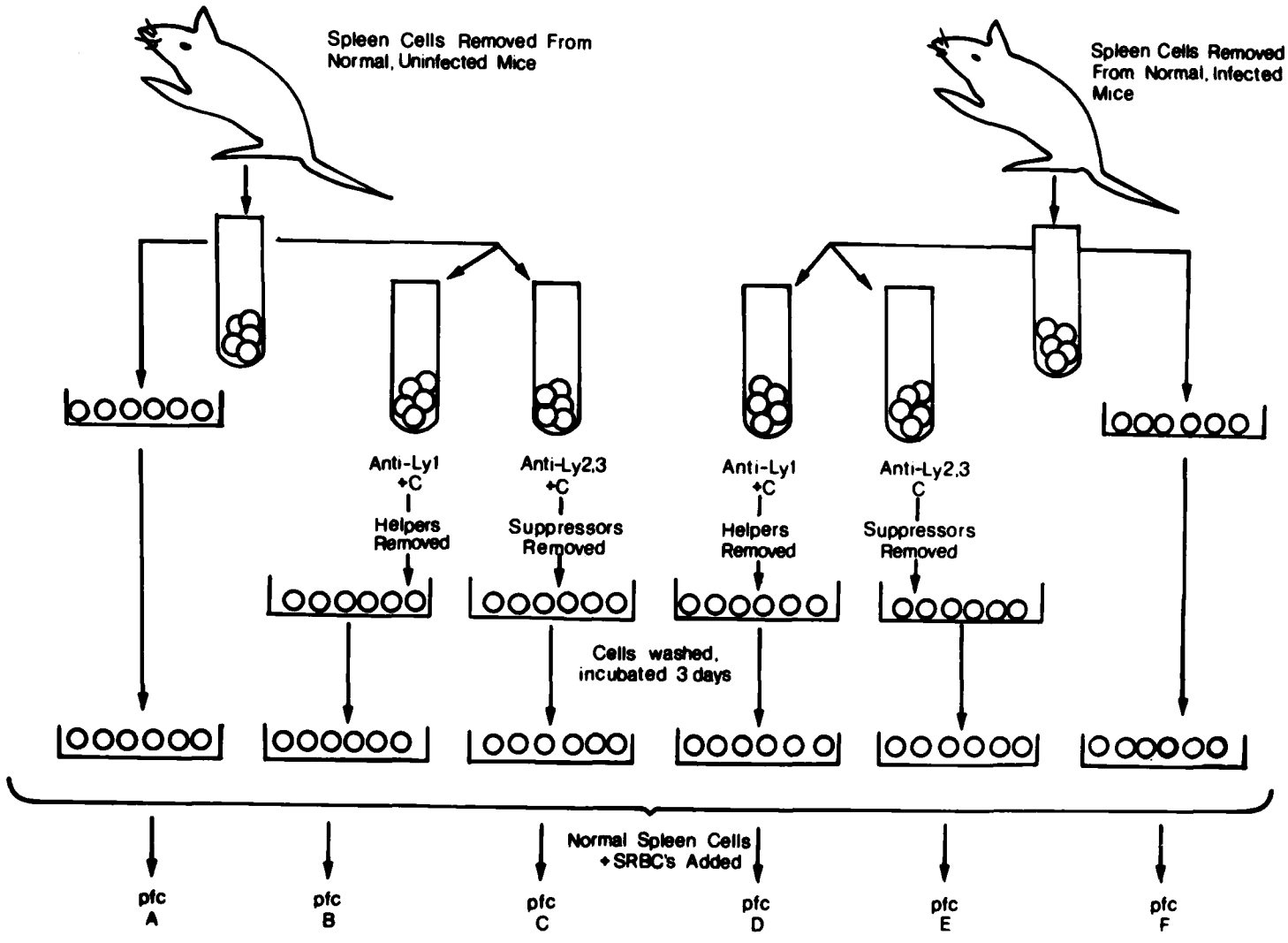


FIGURE 3. Suppressor Cells in Trypanosome Infections

DATA: Figure 4: Suppressor Cells in Trypanosome Infections

Plate Designation	PFC
G	904
H	853
I	422
J	477

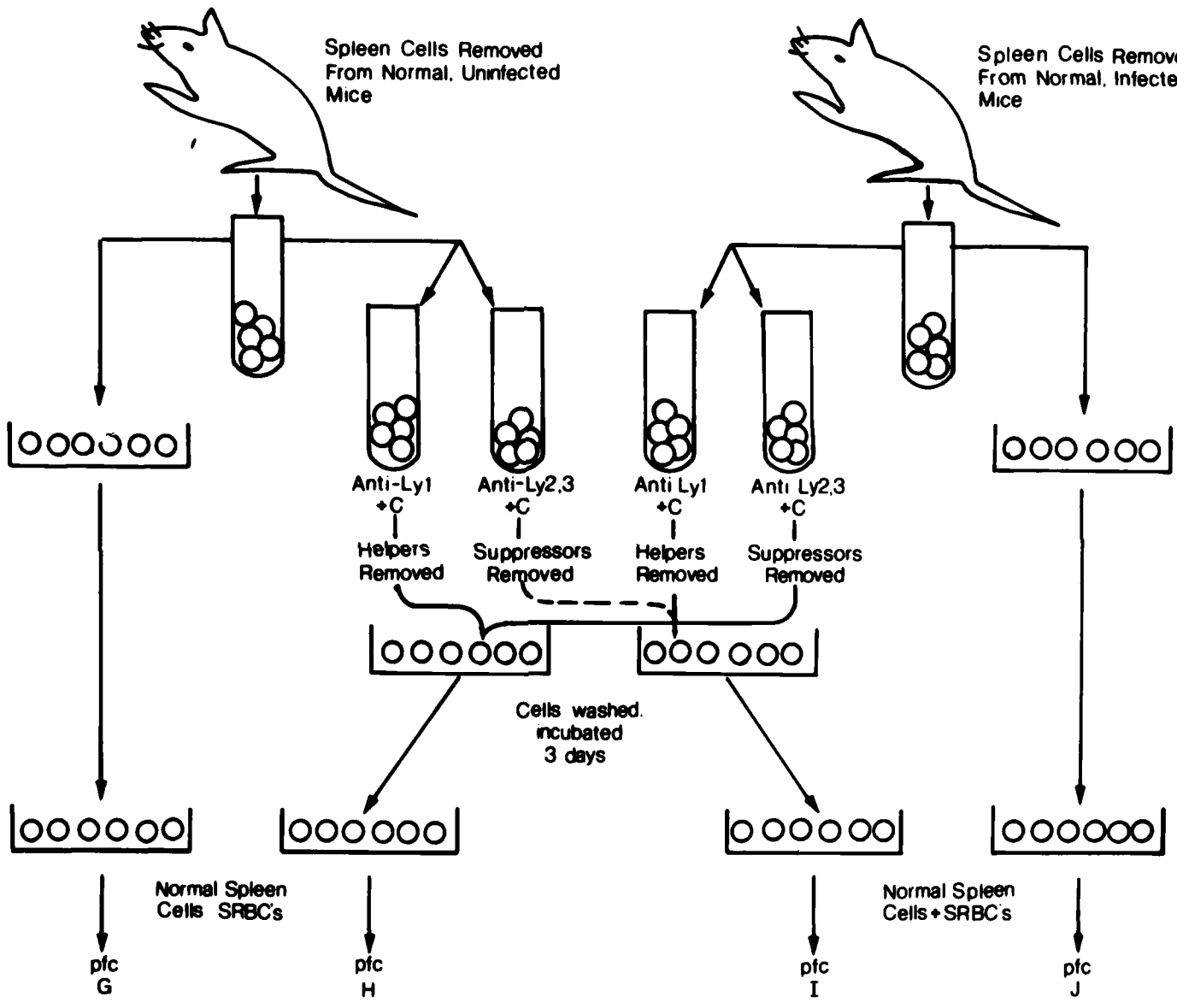


FIGURE 4. Suppressor Cells in Trypanosomal Infections

DATA: Figure 5: Effect of Prostaglandins E_1 and E_2 and PG Inhibitors on Mixed Spleen Cell PFC Response

Plate Designation	Control	(a)	(b)	(c)	(d)
			(conditions)		
A_1	819	-	-	-	-
A_2	-	503	486	781	807
J_1	427				
J_2		465	418	447	453

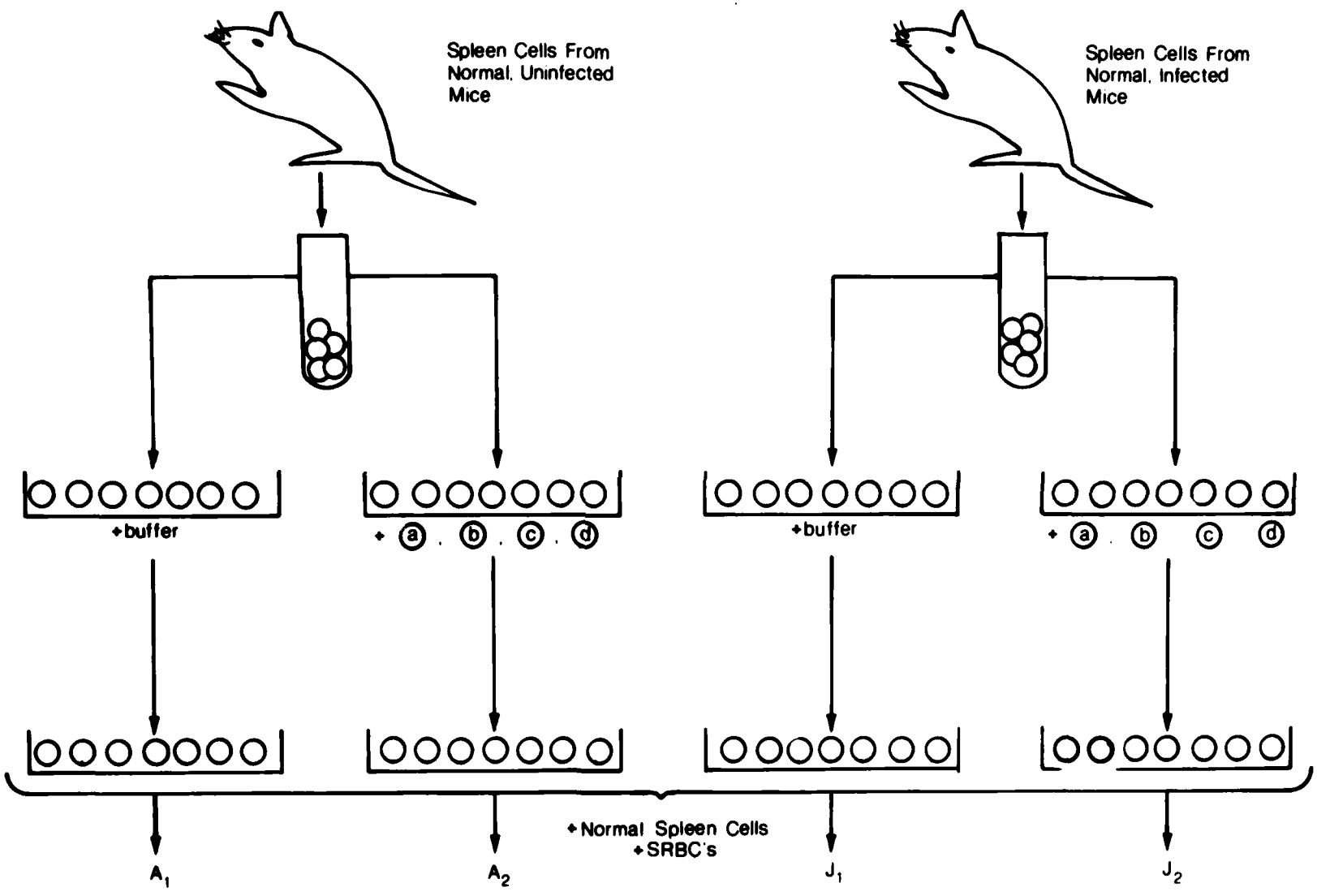
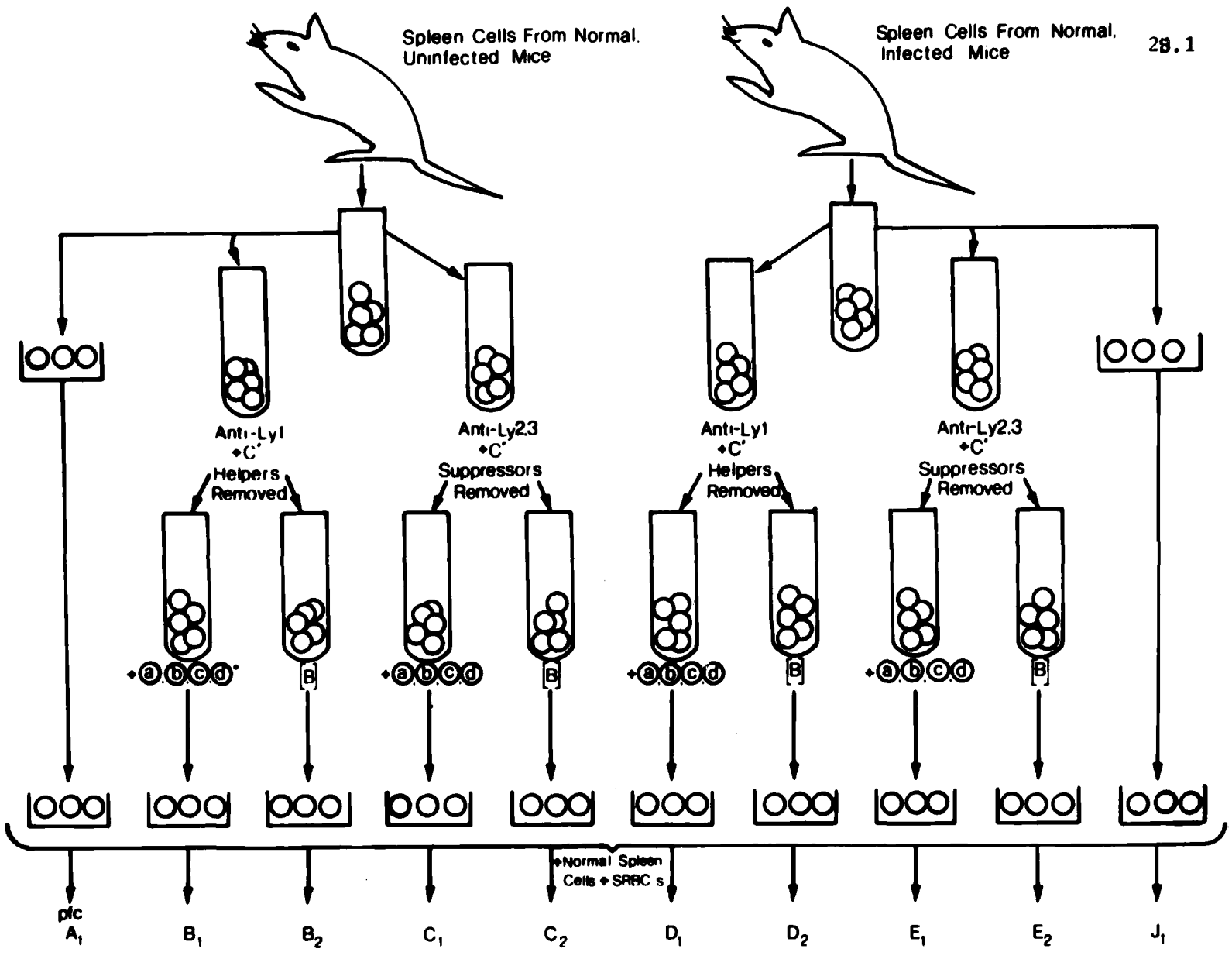


FIGURE 5. Effect of Prostaglandins E_1 and E_2 and PG Inhibitors On Mixed Spleen Cell PFC Response

DATA: Figure 6: Effect of Prostaglandins E_1 and E_2 and PG Inhibitors on Enriched Helper and Suppressor Subpopulations

Plate Designation	PFC (conditions)				
	Control	(a)	(b)	(c)	(d)
A_1	787				
B_1		512	493	706	682
B_2	663				
C_1		418	388	457	469
C_2	855				
D_1		397	405	773	809
D_2	441				
E_1		503	473	426	451
E_2	772				
J_1	379				



*See text for explanation of treatment

FIGURE 6. Effect of Prostaglandins E_1 and E_2 and PG inhibitors on Enriched Helper and Suppressor Subpopulations

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